American Journal of Pathology, Vol. 140, No. 4, April 1992
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Filaments of Lewy Bodies Contain Insoluble Cytoskeletal Elements

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The Lewy body is an intraneuronal inclusion body that is one of the histologic hallmarks of Parkinson's disease, a degenerative disease of the brain. Ultrastructural analysis has shown that the Lewy body is composed of straight 7–20 nm filaments and amorphous elements. Previous light microscopic, immunocytochemical studies have suggested the presence of neurofilament, microtubule, ubiquitin, and paired helical filament–related epitopes in Lewy bodies. Yet the biochemical composition of the Lewy body remains incompletely elucidated. The ultrastructural and immunocytochemical similarities and differences between the Lewy body and the neurofibrillary tangle of Alzheimer's disease raise questions as to their relation to each other and possible shared mechanisms of formation. In this study the authors examine whether ultrastructural immunocytochemical analysis of Lewy bodies confirms the light microscopic data, whether the structures and epitopes of Lewy bodies share with Alzheimer's disease neurofibrillary tangles the property of insolubility in sodium dodecyl sulfate, and speculate about the subunit composition of Lewy body filaments. (Am J Pathol 1992, 140:809–822)

The characteristic cytopathologic hallmark of the neurodegenerative Parkinson's disease is the Lewy body,[1–4] an inclusion body found in the cytoplasm of pigmented neurons in the substantia nigra and locus ceruleus. Lewy bodies were originally observed by Lewy, and named such by Tretiakoff, who also associated them with Parkinson's disease. The Lewy body is composed primarily of filaments,[5,6] a property it shares with the intraneuronal inclusions seen in other neurodegenerative disorders, including the neurofibrillary tangles of Alzheimer's disease and progressive supranuclear palsy, and the Pick bodies of Pick's disease. Extensive light and electron microscopic immunocytochemical studies of these other inclusions (neurofibrillary pathology) have shown epitopes of the proteins comprising the neuronal cytoskeleton,[7–25] unidentified antigens[26–28] and ubiquitin,[10,29–32] all of which localize ultrastructurally to the filamentous components.[9,12,28,29,33] Further, it has been shown that the other inclusions are insoluble in ionic detergents such as sodium dodecyl sulfate (SDS),[29,33,34] which differs from the solubility of the same proteins when located outside of inclusions. These findings have suggested that normal neuronal cytoskeletal components have undergone a transformation at some point while being incorporated into or associated with abnormal filaments.

Light microscopic immunocytochemical analysis of Lewy bodies[22,35–37] has also suggested that Lewy bodies are derived from elements of the neuronal cytoskeleton. These studies have shown that Lewy bodies contain epitopes of neurofilaments,[22,35–37] microtubules,[36] and ubiquitin,[10,32,26] and as yet uncharacterized epitopes shared with paired helical filaments of Alzheimer's disease.[36] The assumption that the filaments of Lewy bodies in Parkinson's disease necessarily contain the epitopes demonstrated by light microscopy is not fully substantiated by these investigations because of the heterogeneous composition of Lewy bodies. Lewy bodies usually have a multilaminated appearance. The lamellae have varying densities, consisting usually of a pale rim and dense central core, which ultrastructurally correspond to zones of filaments versus zones of densely compacted circular profiles,[5] respectively. The densely compacted circular profiles are at least partly lipid in composition.[38] Light microscopic immunocytochemistry alone cannot resolve which Lewy body components contain cytoskeletal or other elements.

In this ultrastructural study, we localize the same components previously shown at the light microscopic level.

Supported by Cuyahoga County Hospital Foundation grant, Akron Children's Hospital Foundation grant, Case Western Reserve University Cancer Center Research grant (30CA43703) (PGG), and NIH grants (AG 07552, AG 09287, and KO4-AG00415) (GP).

Accepted for publication October 25, 1991.

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However, the pattern of staining differed from that expected in some instances, and we speculate as to the cause(s). We show that the filamentous (but not the core) components of Lewy bodies are insoluble in SDS, and that various proteinaceous components associated with the filaments have varying solubilities.

Materials and Methods

Antibodies

The following antibodies were used: 1) monoclonal antibodies recognizing neurofilaments in the phosphorylated (SMI31) (1:100) and 2) nonphosphorylated forms (SMI33) (1:100) (Sternberger Monoclonal, Inc., Baltimore, Maryland), 3) an affinity-purified antiserum to ubiquitin[39] (0.1 mg/ml), 4) a monoclonal antibody to ubiquitin[40] (1:10), 5) an antiserum to MAP2[41] (1:10), 6) an antiserum to paired helical filaments, (ICN)[42] (1:20), 7) an affinity-purified antiserum to tropomyosin[7] (0.05 mg/ml), and 8) an affinity-purified antiserum to τ[43] (0.05 mg/ml).

Tissue Source

For immunogold staining, four autopsied cases of idiopathic Parkinson's disease, age 53, 64, 70, and 76, were used. One of these cases was also used for solubility studies. Two additional idiopathic Parkinson's disease cases, age 54 and 64 years, were also used for solubility studies.

Immunogold Staining

Sections of locus ceruleus and substantia nigra were fixed in 10% formalin in 0.1 M phosphate buffer, pH 7.0. Before use, they were rinsed in phosphate-buffered saline (PBS) and 60 μm free-floating sections were cut with an Oxford Vibratome (Technical Products International, Inc., Earth City, MO). Tissue was dehydrated through graded alcohols and embedded in either Spurr's medium or LR Gold embedding medium. Tissue was sectioned, stained with toluidine blue to localize Lewy bodies, and ultrathinned onto nickel grids. Grids were immunostained using methods previously described.[29] Negative controls were stained with 1% normal goat serum (NGS) in Tris-buffered saline (TBS) in place of primary antibody.

Preparation of Detergent-treated Lewy Bodies

Three cases were from tissue either stored unfixed at −70°C or processed fresh. These were minced onto 0.1 cm^3 pieces and placed into a 1 ml aliquot of 2% SDS in TBS. Controls consisted of locus ceruleus and substantia nigra from the same cases treated with TBS alone. Tissue was treated for either 18 hours (intermediate treatment) or 36 hours (maximal treatment) then sedimented at 5000 g for 15 minutes, dehydrated through graded methanol, then embedded in Spurr's medium or LR Gold. Ultrathin sections were cut throughout the depth of the block onto nickel grids and then immunostained as previously described.[29] Negative controls were stained with 1% NGS-TBS in place of primary antibody.

Sections were contrasted with uranyl acetate and lead citrate after immunostaining and examined at 60 KV on a Zeiss 10 electron microscope.

One of the advantages of a postembedding immunodecoration procedure is that we could compare the decoration produced by different antibodies on the same Lewy body by using serial sections. In most cases, all the antibodies were used to study each Lewy body. Additionally, a given antibody was used to study the same Lewy body at various levels through the serial section.

To give a semiquantitative analysis of the degree of immunostaining in core versus rim, and to assure that Lewy body staining was higher than background, total gold particle counts were made for several Lewy bodies from each group, i.e. intact, intermediately and maximally SDS-treated and TBS-treated. Gold particles were counted in a rectangular area including the core and rim of the Lewy and background outside the Lewy body and standardized per unit area. These counts were directly used in evaluating decoration of areas within a given Lewy body rather than between Lewy bodies.

Lewy Body Filament Diameter Analysis

To assess the degree of solubility of Lewy body filaments, the diameter of 100 filaments per Lewy body was determined in each of three groups 1) intact tissue, and Lewy bodies maximally treated (i.e., for 36 hours) with 2) SDS, or 3) TBS. The average Lewy body filament diameter was calculated for each group and the means were compared by analysis of variance (ANOVA).

Results

Morphology

The Lewy bodies in intact tissue sections revealed the same Lewy body morphology as in previous ultrastructural analyses.[5,6] Lewy bodies with a dense central core consisting of circular profiles and a rim of "radiating" peripheral filaments were observed (Figure 1). Some of the

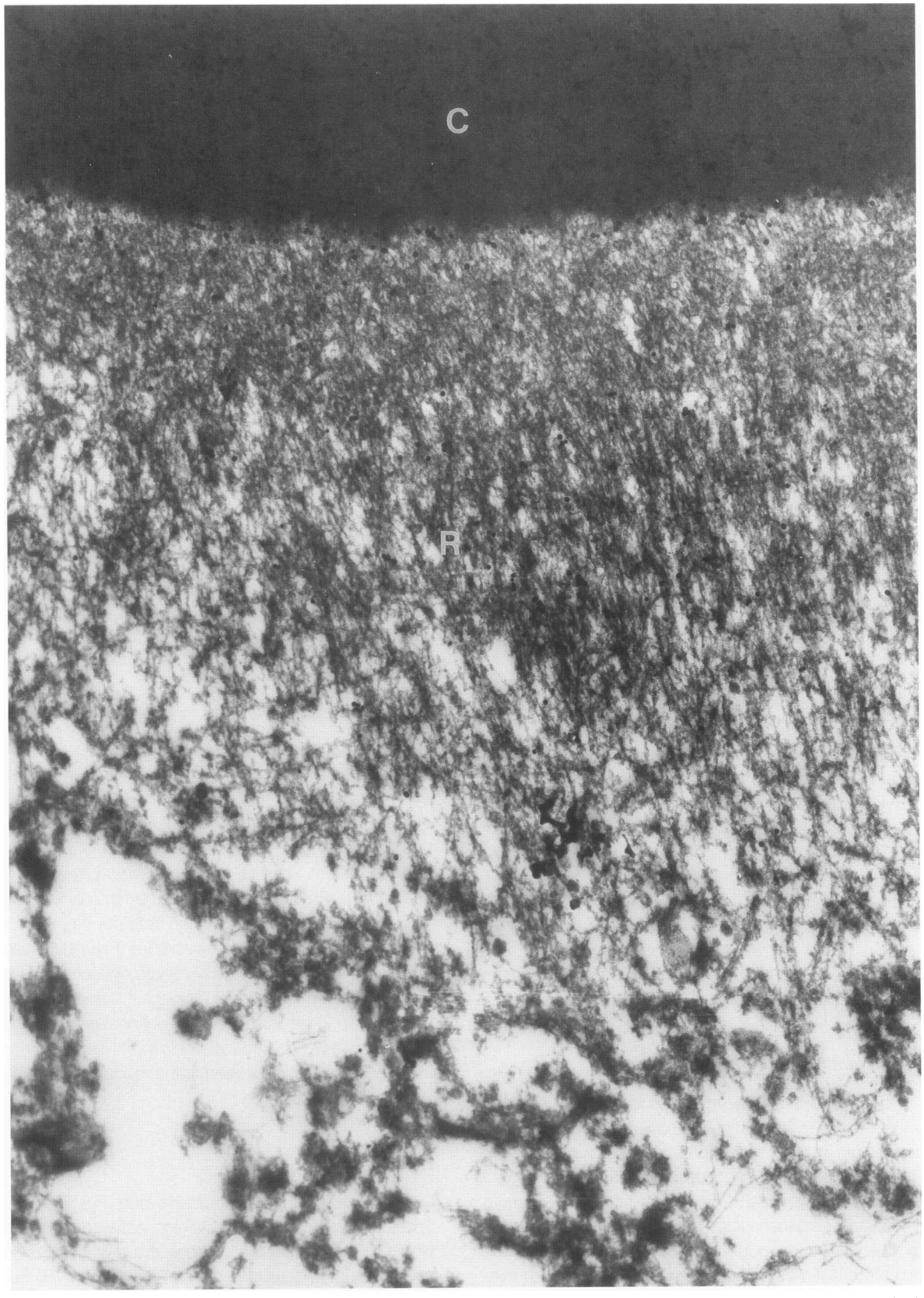

Figure 1. *This Lewy body from intact tissue with a dense central core (C) with circular profiles is immunostained with the ubiquitin antibody, and shows more intense staining in the core than the rim. Note filaments in rim (R) of the Lewy body "radiate" away from the core. Amorphous material is intermixed with the filaments and is more prominent at crossing points of filaments; uranyl acetate/lead citrate, ×40,000.*

dense central cores faded in a stepwise-like manner of intermediate density toward the rim of "radiating" filaments. Some Lewy bodies lacked a dense core of circular profiles entirely, but had a denser packing of filaments in the center compared with the rim (Figure 2A). The discernible filaments both in the center and periphery of the Lewy bodies were partly interspersed with amorphous material (Figures 1, 2A). The amorphous material appeared to be somewhat more prominent or condensed at points where filaments intersected.

Other than somewhat more advanced autolysis, the TBS-treated tissue resembled the intact tissue. Both the intermediate and maximally SDS-treated tissue revealed the presence of Lewy bodies (Figure 4B). In contrast to the TBS-treated tissue, the intermediate SDS-treated tissue revealed dissolution of cell structures, and the maximally SDS-treated tissue revealed total dissolution of cell structures such that only lipofuscin granules and unidentifiable debris were present along with Lewy bodies. Interestingly, the "radial" arrangement of the filaments was less obvious in SDS-extracted preparations. Possibly, this was due to filament rearrangement after removal of the core or that core material at filament intersection points obscures a random arrangement *in situ*. A suggestion of the latter aspect are the filament intersections, apparently violating the "radial" arrangement in the core.

In the intermediate SDS-treated fraction, only scant dark material consistent with the dense central core was seen, whereas no dense central cores were seen in the maximally SDS-treated Lewy bodies. Although no statistically significant difference in filament diameter between groups was demonstrable (see below), the filaments in Lewy bodies in the maximally treated fraction contained markedly less amorphous material around filaments than the TBS-treated fraction (Figure 4B). Since it was not possible to objectively quantitate the number of Lewy bodies or their filament number in each sample, we could not know if all Lewy body filaments were insoluble in SDS.

Immunostaining

Ubiquitin

Immunostaining of the intact tissue (Figure 1) revealed more intense staining in the core than the rim, in contrast to the light microscopic impression of rim staining only. The Lewy bodies in intermediately SDS-treated (Figure 2B) tissue had the same or, in some cases, even more intense staining than intact tissue and TBS-treated Lewy bodies (Figure 2A), suggesting some degree of unmasking of ubiquitin epitopes due to SDS treatment. In contrast, the Lewy bodies in the maximally SDS-treated tissue were unstained (Figure 2C).

SMI31

Lewy bodies in intact tissue (Figure 3A) revealed immunostaining that was essentially confined to the rim. Immunostaining in the core was essentially identical to background, thus confirming the light microscopic impression of rim staining only. Immunostaining in Lewy bodies was less intense than was staining in surrounding cytoplasmic neurofilaments (Figure 3B). Interestingly, in both intermediate- and maximally SDS-treated Lewy bodies (Figure 4A), the core of the Lewy body was stained to a greater extent than the rim, and TBS-treated Lewy bodies had core staining or were unstained. This suggests that SDS-treatment may unmask epitopes, or that the neurofilament epitopes in the rim may be more liable to postmortem autolysis.

SMI33

In intact tissue (Figure 5A) both the rim and core were stained, with the rim slightly more heavily decorated than the core. This result differs from the interpretation of previous light microscopic studies, in which the inner and outermost borders of the filamentous array were believed to be stained, and the core unstained. SDS-treated Lewy bodies (Figure 5B) revealed primarily rim staining, suggesting the epitopes in the core may be soluble.

Paired Helical Filament

Intact tissue sections revealed approximately equal immunostaining in the Lewy body core and in the rim, differing from the light microscopic impression of staining of the inner and outermost borders. The TBS-treated Lewy bodies (Figure 6A) either had rim-only staining or a staining pattern identical to intact tissue. Lewy bodies treated with TBS for 36 hours had the rim-only staining, suggesting loss of epitopes in the core due to postmortem autolysis. SDS-treated Lewy bodies (Figure 6B) were unstained, suggesting the PHF-related epitopes in Lewy bodies in the rim are soluble. Although this antiserum has been shown to recognize τ, since antibodies to τ do not recognize Lewy bodies (see below), its decoration of Lewy bodies must be related to the recognition of other unidentified epitopes.

MAP2

Lewy bodies from TBS-treated tissue (Figure 7A) revealed approximately equal staining in the core and rim, which differs from the light microscopic impression of rim only staining. Those treated with SDS (Figure 7B) had diminished staining compared with TBS-treated, suggesting some solubilization of epitopes.

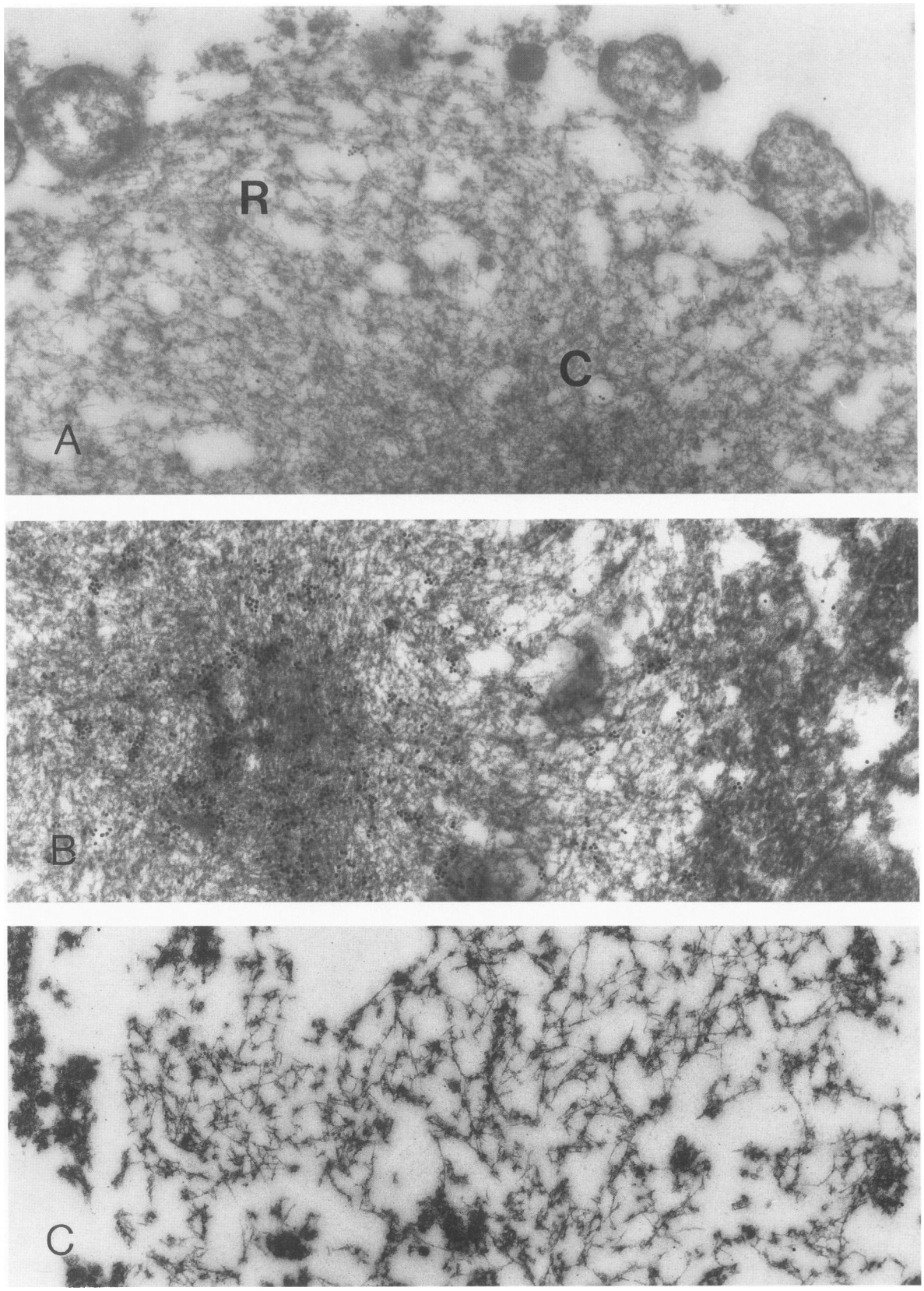

Figure 2. **A:** *After TBS treatment, the Lewy body core (C) region still maintains more ubiquitin epitopes than the rim (R). The core of this Lewy body consists of a denser central packing of filaments, rather than circular profiles.* **B:** *Intermediately SDS-treated Lewy bodies immunostained with the ubiquitin antibody.* **C:** *Lewy bodies from the maximally SDS-treated fractions did not show any gold decoration when immunostained with the ubiquitin antibody; uranyl acetate/lead citrate,* **A,** *×27,000;* **B,** *×21,000;* **C,** *×21,000.*

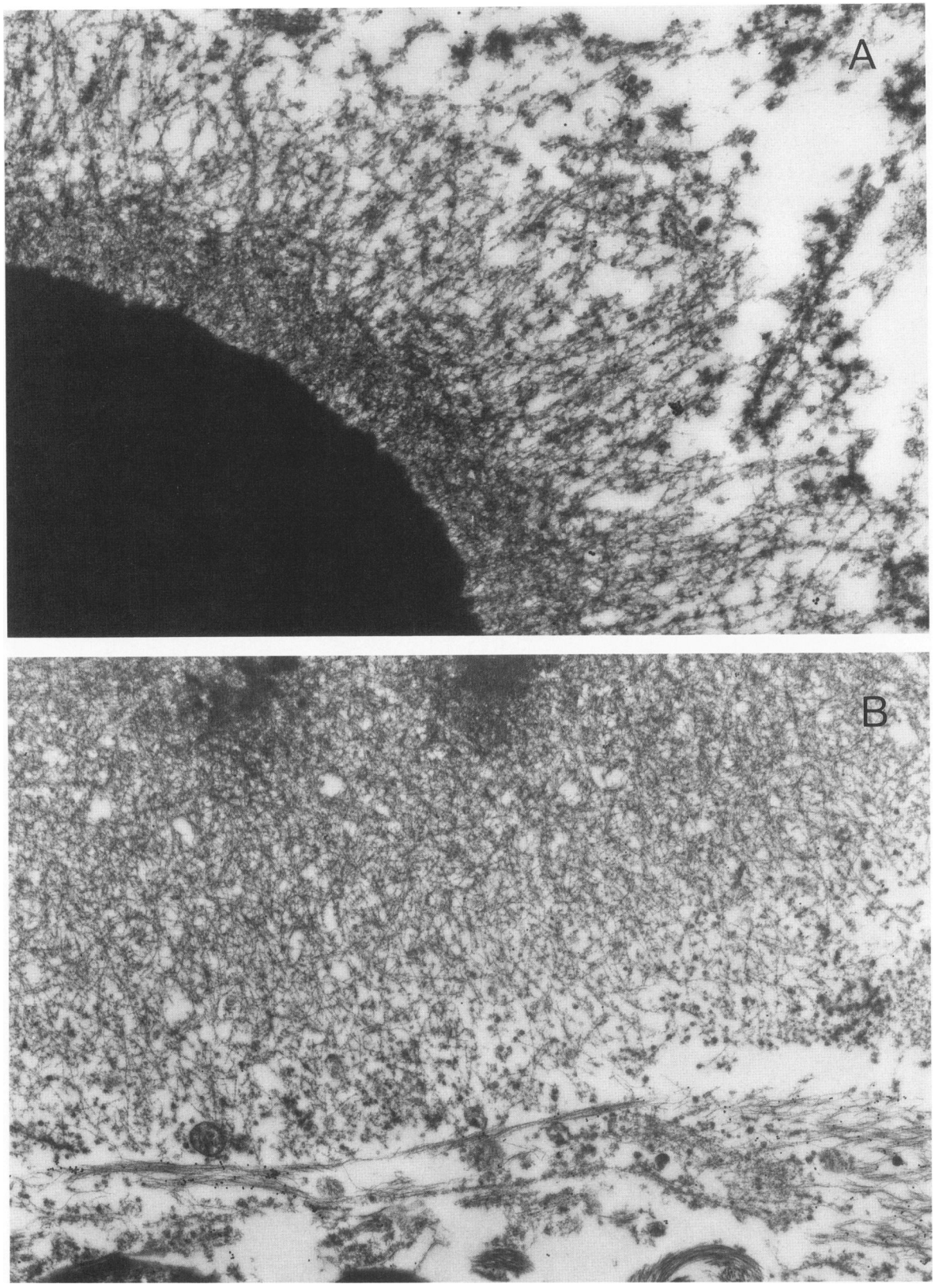

Figure 3. A: *Immunostaining of Lewy bodies in intact tissue with the phosphorylated neurofilament antibody SMI31 gave weak decoration essentially confined to the rim.* B: *This weak decoration contrasts with that produced by the same antibody for neurofilaments in the surrounding cytoplasm; Lewy body (upper part of field) and neurofilaments (lower part of field); uranyl acetate/lead citrate,* A, *×23,000;* B, *×12,000.*

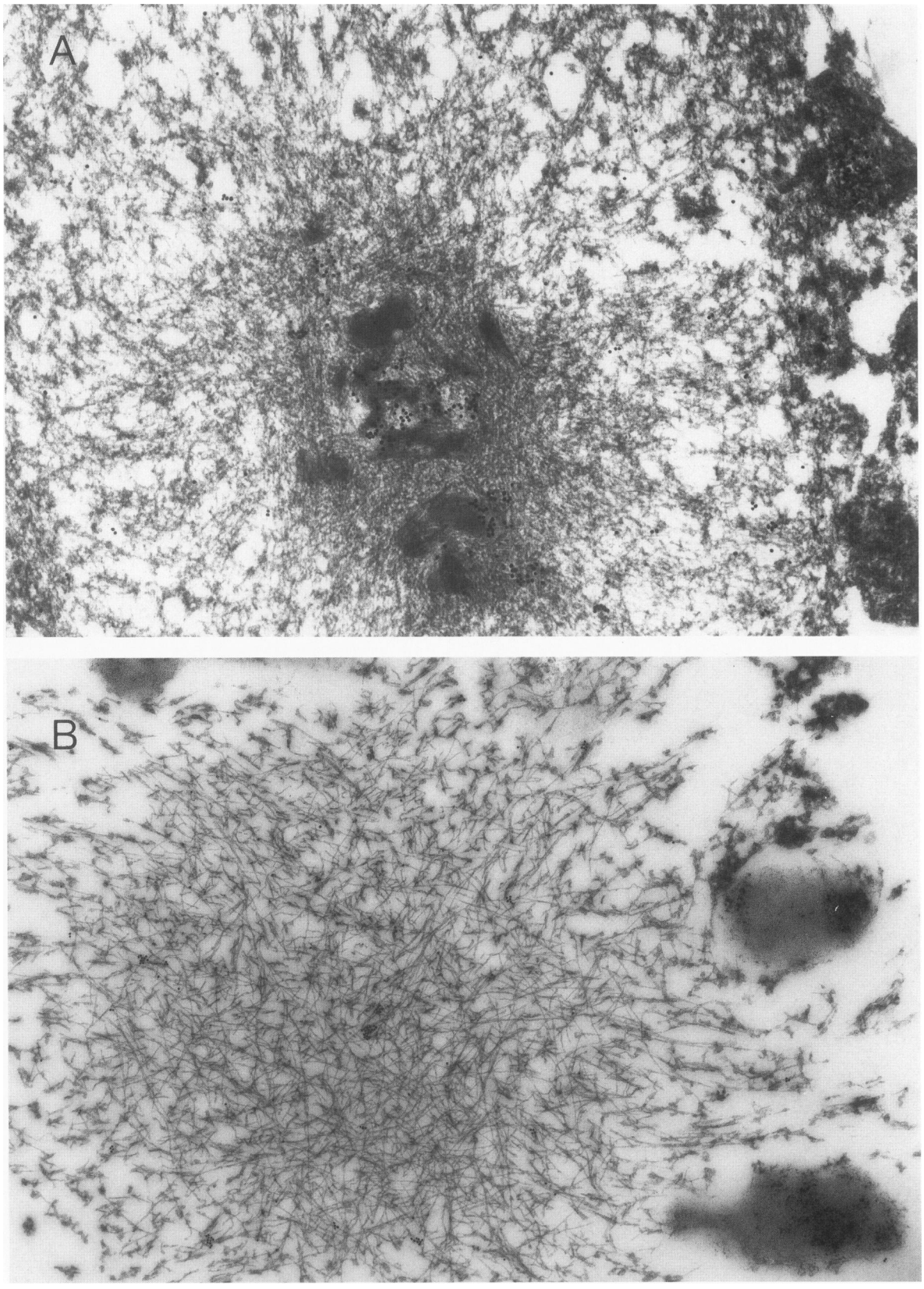

Figure 4. **A:** *The Lewy body in the intermediately SDS-treated preparation showed greater core than rim staining with the phosphorylated neurofilament antibody SMI31.* **B:** *A Lewy body remaining after 36 hr in SDS. The only other material present was lipofuscin and amorphous debris (surrounding the Lewy body in the field). There is slightly greater gold decoration in the core of the Lewy body than in the rim in this Lewy body immunostained with the SMI31 antibody. Note diminished amount of amorphous material compared to non-SDS extracted Lewy bodies; uranyl acetate/lead citrate,* **A,** *×30,000;* **B,** *×21,000.*

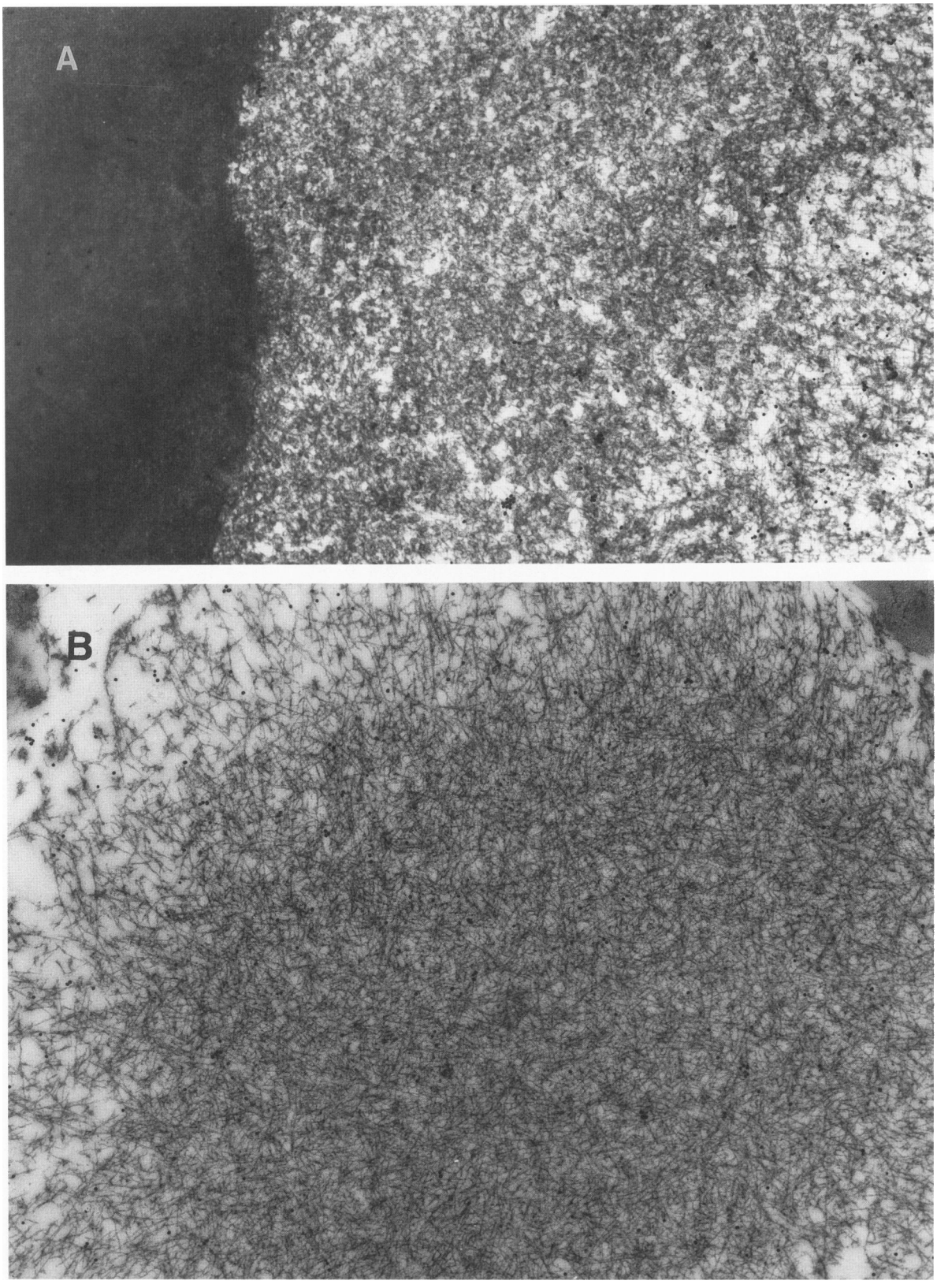

Figure 5. A: *Both the rim and core of this Lewy body in intact tissue are immunodecorated with the antibody to nonphosphorylated neurofilaments, SMI33.* B: *Lewy body maximally treated with SDS. Immunostaining with SMI33 showed more decoration at the rim, whereas no dense core of circular profiles is noted in the center. A greater density of filaments is seen in the center after SDS; uranyl acetate/lead citrate,* A, ×*25,000;* B, ×*24,000.*

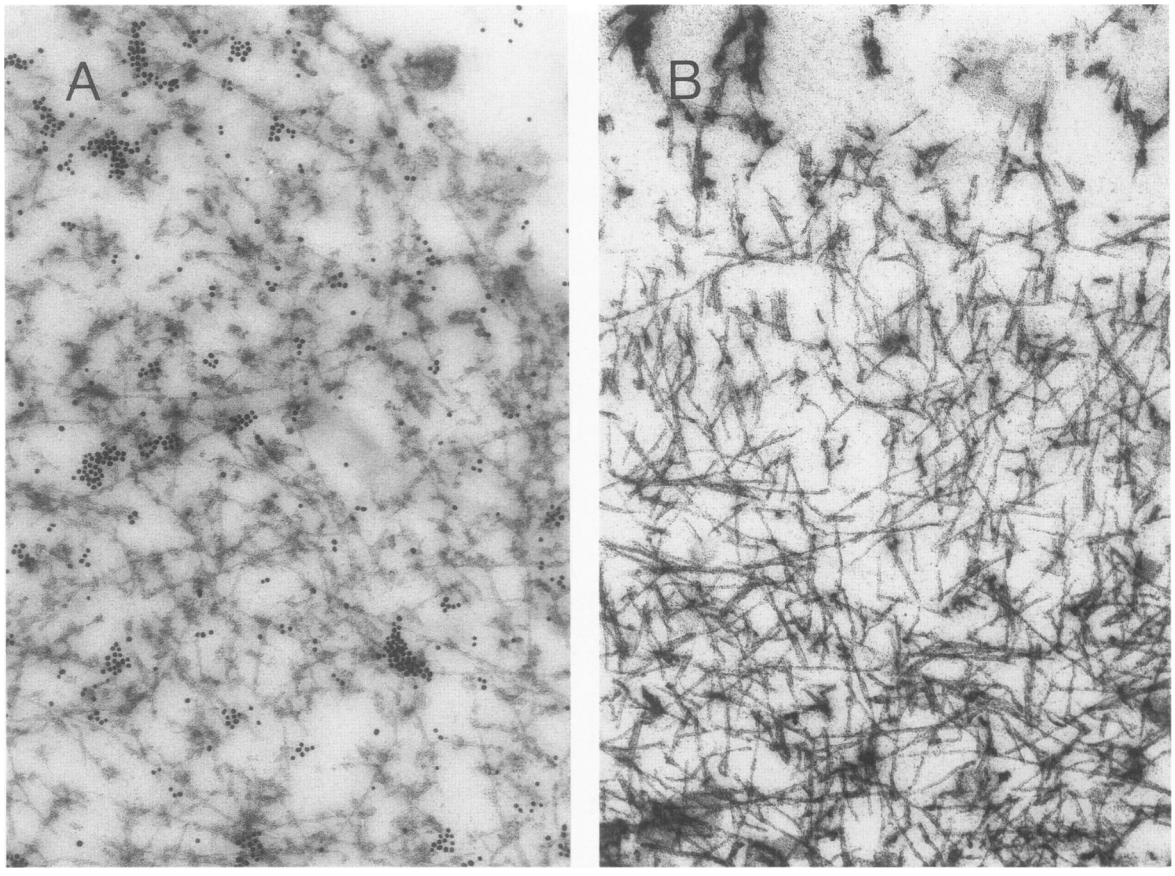

Figure 6. **A:** *Lewy body with diffuse gold decoration produced by the antiserum to paired helical filaments.* **B:** *The epitopes for this antiserum are removed by maximally SDS-treatment; uranyl acetate/lead citrate;* **A,** × *47,500;* **B,** ×*50,500.*

τ and Tropomyosin

Lewy bodies were unstained.

Negative Control

Lewy bodies were unstained (Figure 8A,B).

Statistical Analysis

The average filament diameter for the following groups was observed: intact tissue, 13.1 nm; TBS-treated, 13.4 nm; SDS-treated, 13.9 nm. By ANOVA, the differences were not significantly different at the 0.05 level. The Lewy body filament diameter is thicker than normal neurofilaments (7–10 nm).

Discussion

This ultrastructural study shows that the filaments of at least some Lewy bodies, but not the core, are insoluble in SDS. The only structure that might have been confused with the Lewy body filaments are axonal spheroids, yet there are clear distinctions. First, the Lewy body filaments seen are consistently 13 nm in diameter whereas neurofilaments are thinner. Second, the Lewy body filaments are not as intensely decorated as neurofilaments are by antibodies to neurofilaments (Figure 3B). Third, neurofilaments are known to be soluble in SDS. Finally, neurofilaments in axonal spheroids are spiral-like. Lewy bodies share the property of SDS insolubility with the filaments and cytoskeletal elements found in inclusions of other neurodegenerative diseases, which are also predominantly insoluble. Also similar to MAP2 and some neurofilament antigens in neurofibrillary tangles;[44,45] ubiquitin and MAP2 epitopes in Lewy bodies are soluble in SDS. The phosphorylated and nonphosphorylated neurofilament epitopes were most resistant to SDS-treatment suggesting that neurofilaments are greatly altered during formation of the Lewy body. The difference between the degree of staining of Lewy bodies and normal neurofilaments (Figure 3B) in the same section suggests either that epitopes are modified or that there may be other as yet unidentified proteinaceous components, possibly unique to the Lewy body. Also, Lewy body filaments may, like PHF, be heterogeneous and after SDS some filaments might have been removed.

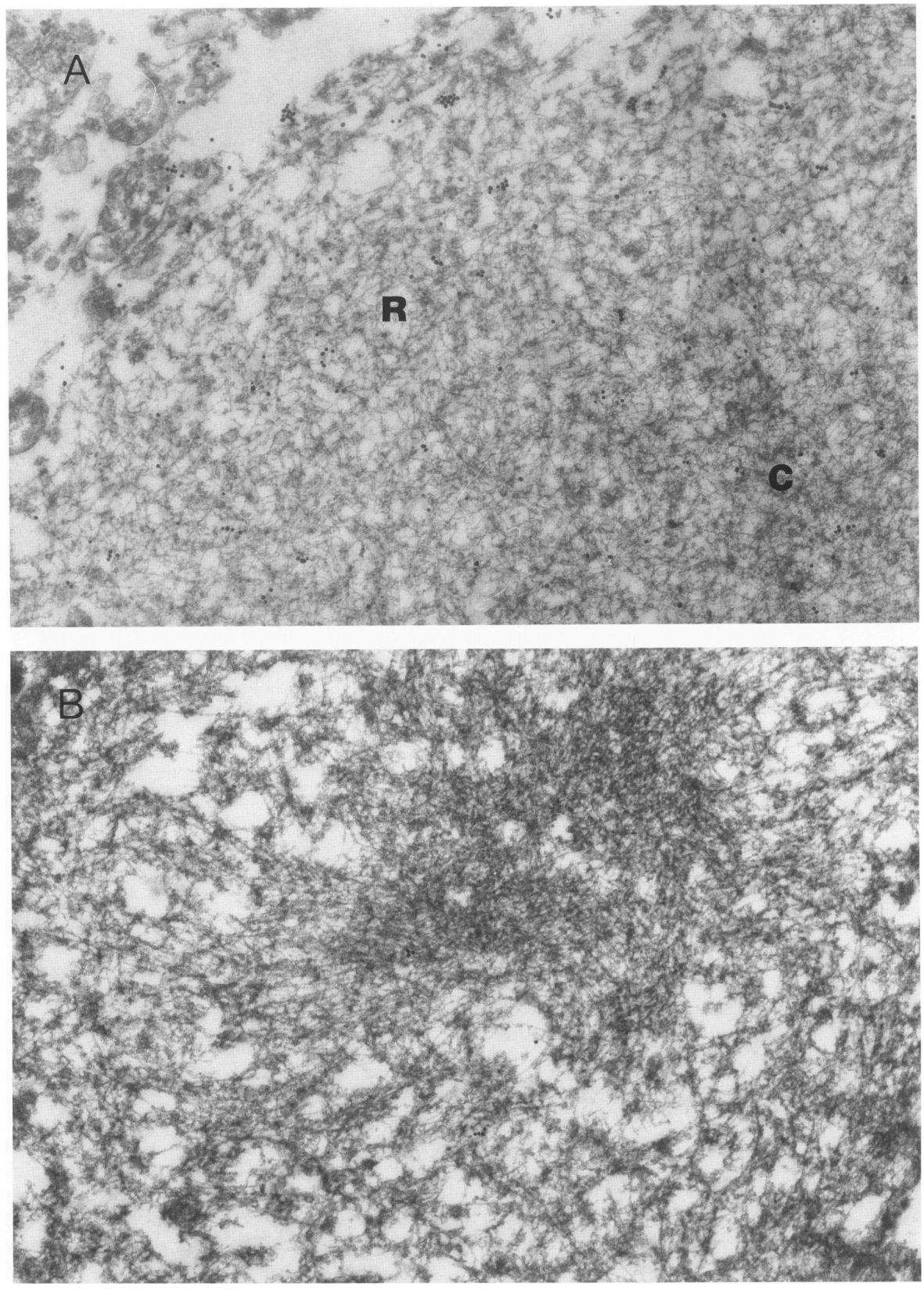

Figure 7. **A:** *The MAP2 antibody yielded approximately even staining in core (C) and rim (R) regions of TBS-treated Lewy bodies.* **B:** *After SDS-treatment decoration was reduced; uranyl acetate/lead citrate,* **A,** *×29,000;* **B,** *×33,000.*

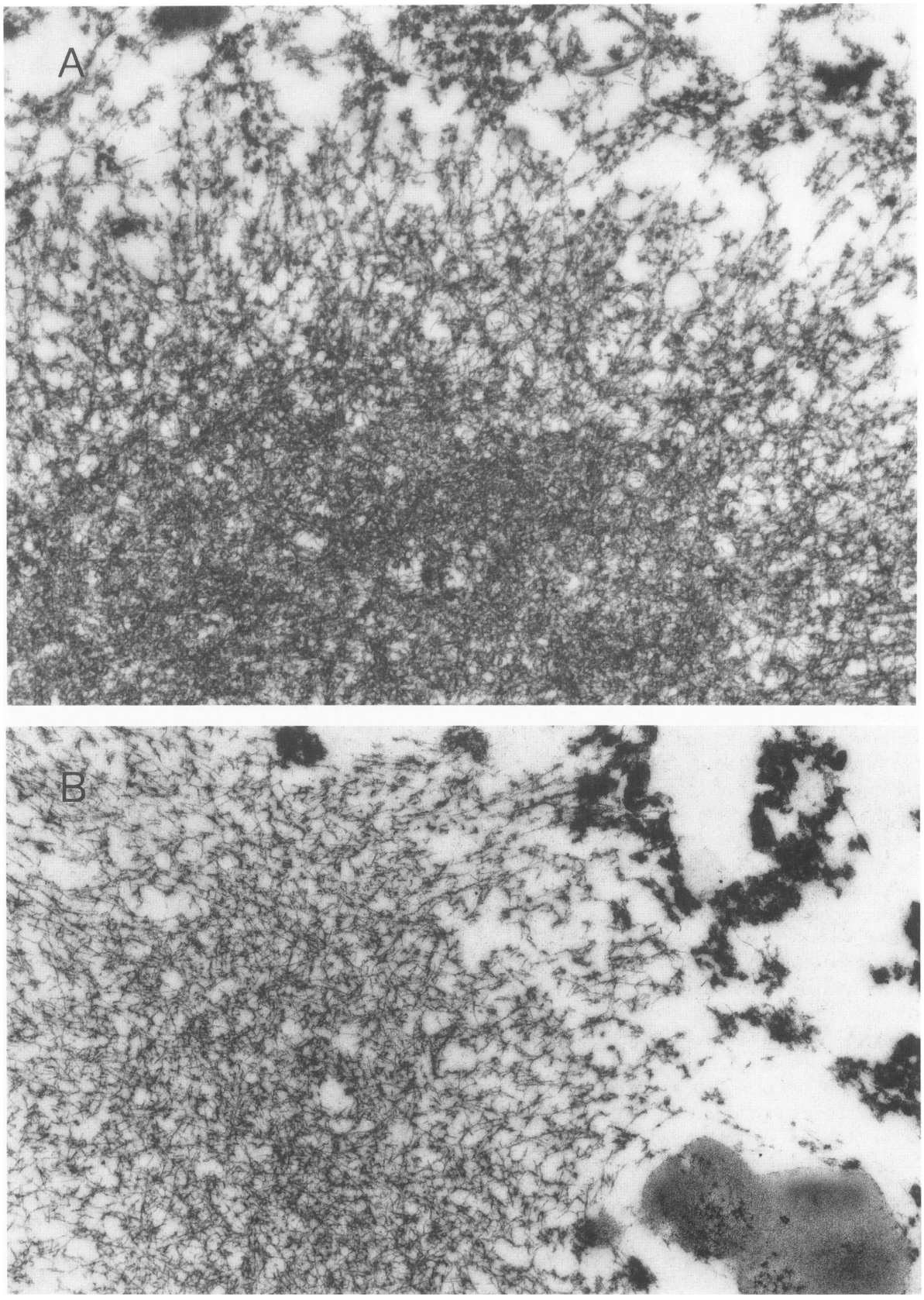

Figure 8. *When the 1% NGS-TBS was used in place of primary antibody, no gold decoration was observed.* **A**: *Lewy body from intact tissue.* **B**: *Lewy body after maximally SDS-treatment; uranyl acetate/lead citrate,* **A, B,** *×21,000.*

Another aspect of this study is a difference between the light microscopic impression of immunostaining and those observed ultrastructurally, suggesting that the light microscopic data were misinterpreted. In contrast to the light microscopic interpretation in which the core of the Lewy body appeared unstained, immunostaining was demonstrated in the core ultrastructurally with the SMI33, PHF, ubiquitin, and MAP2 antibodies. Perhaps in paraffin sections, the epitopes are partially or totally masked by other components, but in our postembedding preparations the antibodies have equal access to all Lewy body components in the plane of section. Staining of neurofilament epitopes in the partially solubilized core of the Lewy body would tend to support the concept that these epitopes are masked in some way, rather than missing.

The other difference is that in our previous study[36] the PHF and SMI31 antibodies stained in a pattern in which reaction product appeared to be deposited in the inner and outermost borders of the rim which probably corresponds to the filamentous array. This staining pattern was not confirmed by ultrastructural analysis. The most probable explanation for the difference is related to the marked heterogeneity of Lewy body morphology. Although the classic appearance of the Lewy body by electron microscopy is that of circular profiles in the center, with "radiating" filaments at the periphery, some Lewy bodies have been shown to have multiple concentric rings of circular profiles alternating with the filamentous material.[5] If a Lewy body with such an arrangement were immunostained light microscopically using the PHF and SMI31 antibodies, and access was only gained to the filamentous component and not to the lipid component, the light microscopic pattern would appear to be the inner and outermost borders of the filamentous array. Thus, the light microscopic appearance of rim staining may be an artifact of epitope accessibility.

This suggests a parallel with an earlier study of Hirano bodies, in which the initial light microscopic results suggested that actin epitopes were condensed at the periphery whereas ultrastructural analysis[47] revealed the epitopes were distributed throughout the filaments composing the body but were only stained in areas accessible to antibodies.

One previously unresolved aspect of the structure of the Lewy body is the composition of the core. The cores of Lewy bodies in intact tissue and partially SDS-treated tissue are immunostained, suggesting the core at least partially contains nonphosphorylated neurofilament, paired helical filament, ubiquitin, and MAP2 epitopes in addition to lipid-related components.[38]

The lack of immunostaining with the tropomyosin and τ antibodies that was observed with light microscopy[36,48] was confirmed ultrastructurally. The absence of τ further distinguishes the Lewy body from other neurofibrillary pathology. The lack of demonstrable τ suggests that τ may not be essential for Lewy body formation, or that it is present in filaments (as opposed to amorphous debris) in an antigenically inaccessible form.

Previous immunocytochemical studies of neurofibrillary pathology have suggested that the filaments are composed of altered brain proteins,[8] and that the major component may be τ.[49] The results of our study suggest that the Lewy body filaments may be primarily composed of 1) as yet unidentified, markedly altered proteins, 2) markedly altered neurofilaments, or 3) a nonproteinaceous component. If neurofilaments, whether phosphorylated or nonphosphorylated, are the principal constituent of Lewy body filaments, they must be markedly altered to resist solubilization by SDS.

Acknowledgments

The authors thank Mrs. Elvira Occhipinti and Ms. Sandra Bowen for preparing the manuscript; Ms. Teri Hnat, Mrs. Kathy Schlosser, and Mrs. Igna Cioletti for assisting with electron microscopy; Dr. Arthur Haas for providing the ubiquitin antibody; and Mrs. LuAnn Sadowski for helping with statistical analysis.

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